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ORIGINAL PAPER

Overexpression of Cdk5 or Non-phosphorylatable Retinoblastoma Protein Protects Septal Neurons from Oxygen–Glucose Deprivation

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Abstract Activation of cyclin dependent kinases (Cdks) contributes to neuronal death following ischemia. We used oxygen–glucose deprivation (OGD) in septal neuronal cultures to test for possible roles of cell cycle proteins in neuronal survival. Increased cdc2-immunoreactive neurons were observed at 24 h after the end of 5 h OGD. Green fluorescent protein (GFP) or GFP along with a wild type or dominant negative form of the retinoblastoma protein (Rb), or cyclin-dependent kinase5 (Cdk5), were overexpressed using plasmid constructs. Following OGD, when compared to controls, neurons expressing both GFP and dominant negative Rb, RbΔK11, showed significantly less damage using microscopy imaging. Overexpression of Rb-wt did not affect survival. Surprisingly, overexpression of Cdk5-wild type significantly protected neurons from process disintegration but Cdk5T33, a dominant negative Cdk5, gave little or no protection. Thus phosphorylation of the cell cycle regulator, Rb, contributes to death in OGD in septal neurons but Cdk5 can have a protective role.

Keywords cdc2 · Retinoblastoma · Cdk5 · Ischemia · Transfection · Neuron

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Introduction

Inappropriate activation of cell cycle related proteins in post-mitotic neurons is hypothesized to contribute to neuronal death [1]. Activation of cell cycle related proteins is believed to contribute to the delayed neuronal death following ischemia [2–4]. Increased cell cycle protein expression in neurons after cardiac arrest or cerebral infarction has been reported in post-mortem human brain [5]. Studies in animal models have also shown an increase in cyclins or a decrease in CDKIs following ischemic stress [6, 3, 7]. Moreover, pharmacological blockade of Cdk4 and Cdk6 has also been reported to be neuroprotective after ischemia [8].

Three predominant families of proteins are involved in the execution of cell cycle functions: (1) cyclins, (e.g. cyclin D, E, A), (2) cyclin-dependent kinases (Cdks; e.g. Cdk2, Cdk4, Cdk6)and (3) cyclin-dependent kinase inhibitors (CdkIs) [9]. The cyclins form active complexes with the cyclin-dependent kinases, which then phosphorylate substrate proteins thereby allowing progression through different phases of the cell cycle. One mechanism by which post-mitotic neurons enter the cell cycle is through activation (phosphorylation) of retinoblastoma protein (Rb) by cyclinD/Cdk4 complex [9, 10]. Rb in its hypophosphorylated form is a negative regulator of cell cycle progression (see [11] for review). Upon phosphorylation, Rb dissociates itself from E2F transcription factors [12] which are responsible for the entry of cells into S-phase.

Recent studies have also implicated Cdk5 in the death of neurons following various stresses including ischemia [13–15]. Cdk5 is a serine/threonine kinase that is not primarily involved in cell cycle control functions although it can phosphorylate Rb in SH-SY5Y neuronal cell line [16]. Cdk5 is expressed in most tissues, but its kinase activity is especially prominent in the brain [17]. Cdk5 is activated by its binding to a neuron-specific activator p35 or its isoform p39, and as a monomer it has little kinase activity [18, 19]. Cdk5 regulates neurite outgrowth and several intracellular processes including cytoskeletal dynamics [20] and cortical development [21]. It has been suggested that following stress the regulatory protein p35 is cleaved to p25 by calpain, a calcium-dependent protease, and the abnormal activation of Cdk5 by p25 is neurotoxic [22, 23]. While blockade of Cdk5 is believed to be neuroprotective in ischemia [13, 23], it is not known if overexpressing Cdk5 before ischemic stress can ameliorate or exacerbate neuronal injury.

The present study used septal neurons; these and other basal forebrain neurons are damaged during stroke-induced dementia [24]. We demonstrate that a cell cycle-regulated protein kinase cdc2 (also called Cdk1) is up-regulated in cultured rat septal neurons following OGD. We tested whether transfection with constructs for wild-type and dominant-negative forms of Rb or Cdk5 would affect neuronal survival following OGD, and report that survival is enhanced by dominant-negative Rb and by wild-type Cdk5. Thus for septal neurons exposed to this in vitro model of ischemia, Cdk5 appears to be neuroprotective.

Experimental Procedure

Neuronal Cell Culture

Neuron-rich cultures were prepared as described in Nonner et al. [25]. Briefly, septal regions were dissected from embryonic day 15 (E15) Sprague–Dawley rats, dissociated by gentle trituration and plated in Neurobasal medium supplemented with an acid-stable 55 kDa fraction of horse serum (1 mg protein/ml, [26]) which contains selenoprotein-P [27]. Cultures were plated in poly-L-lysine coated Nunc Terasaki microwell plates. Cells were maintained at 37°C in 95% air/5%CO_2. Procedures followed guidelines established by the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the local animal care committee (IACUC) of the University of Miami, Miami, FL. For preparation of neuronal cultures, pain and suffering of animals were minimized by use of anesthetics as approved by IACUC.

Immunohistochemistry

Cultures were fixed with 4% paraformaldehyde for 30 min, blocked with 10% goat serum (Jackson ImmunoResearch, West Grove, PA, USA) for 30 min, and incubated overnight with rabbit anti-p34cdc2 (1:1000; Santa Cruz Biotechnology, CA) and subsequently with Alexa Fluor 647-labeled goat anti rabbit IgG (1:2000; Molecular Probes). To check for non-specific binding, cultures in each experiment were incubated without the primary antibody.

Cell Counting Protocol

Cell counts were done as described previously [28]. Briefly, to determine the total number of cells in each well, Hoechst (bisbenzimide) 33,342 (16 μM, Molecular Probes, Eugene, OR, USA) was added to the cultures to label the nuclei of all cells. The fluorescence signals from these dyes were imaged using a 10× microscope objective using a Leica DMIRBE microscope equipped with a monochrometer and CCD camera (ORCA-ER, Hamamatsu Photonics K.K., Hamamatsu-City, Japan). Each field encompassed the entire Terasaki plate microwell (1.3 mm^2) so that majority of cells in that well was counted. Imaged fields were automatically selected by a computer controlled OptiScan motorized microscope stage (Prior Scientific Instruments, Rockland, MA, USA). Two separate images of each well were taken using excitation and emission wavelengths appropriate for each dye: excitation 380 nm/emission 450 nm for Hoechst, and 635/668 nm for Alexa Fluor 647. The total number of cells in each microwell was measured by counting the number of Hoechst-positive cells. A computer macro written in V++ imaging language (Digital Optics, Browns Bay, Auckland, New Zealand) was used to count the number of fluorescent cells in each image. The macro counted a Hoechst-stained object as a nucleus if it had a minimum diameter of at least 2 μm with pixel values exceeding five times the SD of the background. For cdc2-immunopositive cells, the macro (in V++) constructed histograms of the number of cells with a given fluorescence intensity. Cells with fluorescence intensity values exceeding five times the SD of the background was counted. Manual counts on some of the images were also done as a check and yielded results within 5% of those computed by the macro.

Transient Transfection

The following plasmids were used in the study: Green fluorescent protein (pIRES-2EGFP, Clontech, Palo Alto, CA), HA-tagged retinoblastoma-wild-type and mutant (pGL3, gift from Dr. Eldad Zacksenhaus, Toronto General Hospital, Canada), pEGFPCdk5 (wild type) and pEGFP-Cdk5T33 (dominant negative; pcDNA3.1); gift from Dr. Zelenka (NIH, USA; [29]). Mutant Rb (Rb.K11) has 16 potential phosphoacceptor sites mutated and can not be fully phosphorylated [30]. Cdk5T33 has no kinase activity, owing to a K to T substitution at amino acid 33 [31], and exerts its dominant negative effect by sequestering p35. Plasmid amplification and subsequent DNA quantitation methods are described elsewhere [32].

Neurons (10–12 days in vitro) were co-transfected with plasmids containing cDNA for GFP along with Rb-wt or Rb.K11 or with GFP-Cdk5wt or GFP-Cdl5T33 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Plasmid DNA concentration did not exceed 0.05 mg/1,000 cells. Following transfection cultures were washed with medium 24 h later, and then fed with a mixture (1:1) of normal medium and neuronal conditioned medium. Experiments were conducted 48 h following transfection. The transfection efficiency was between 5% and 8%, and all experiments were repeated with multiple platings from different seedings.

Imaging of Transfected Neurons

Transfected neurons containing GFP were imaged using a Leica DMIRBE fluorescence microscope as described previously [32]. Briefly, an incubator chamber on the microscope maintained CO_2 and temperature during imaging. The use of microwell cultures together with a computer-controlled microscope stage enabled us to follow the same transfected cells over 72 h. GFP-transfected neurons were imaged immediately before OGD and 24–48 h after end of OGD. In the absence of any stress, transfected cells maintained normal morphology with a well-defined cell body and intact neuronal processes. Survival of transfected neurons was determined by counting the number of fluorescent neurons that had intact morphology and did not stain for propidium iodide, a marker for dying or dead cells.

Oxygen–Glucose Deprivation

OGD was performed as described in Panickar et al. [32]. Briefly, regular Neurobasal medium was replaced with hypoglycemic medium consisting of normal salts [33], 1 mg/ml bovine serum albumin, 1 mM glucose (regular medium contains 25 mM glucose) and 10 mM 2-deoxy-glucose (to limit glucose uptake and glycolysis). Cultures were then exposed to 95% nitrogen, 5% CO_2 atmosphere maintained by constant gas flow at 37°C for 5 h in all transfection experiments. Images were taken of each culture well just before the switch to OGD and also at 24 and 48 h after end of OGD. Control cultures were imaged and then the medium was replaced with new standard Neurobasal medium.

Statistical Analysis

Data are presented as mean ± SEM for the indicated number of culture wells. Each set of transfection experiments was repeated in 2–3 different platings. Paired comparisons used Student's two-tailed t-test. Comparisons of multiple conditions with a common control or treatment group were done using analysis of variance (ANOVA) followed by Scheffe's post-hoc test.

Results

Increase in cdc2-Immunoreactivity in Septal Neurons after OGD

Septal neuronal cultures (14 DIV) plated in 72-well culture plates were subjected to OGD for 5 h. At 24 h after the end of OGD cells were processed for cdc2 protein levels immunohistochemically. cdc2 is a cell cycle-regulated protein kinase that complexes with cyclin B to form an active heterodimer. OGD produced a 156% increase in the number of cdc2-immunoreactive cells when compared to controls ($P < 0.001$; Fig. 1). In control cultures cdc2 appeared to be only in the cell body; after OGD, immunoreactivity was also observed in neurites of some neurons (Fig. 1).

Overexpression of Non-phosphorylatable Rb Prevents Disintegration of Neuronal Processes Following OGD

Since most pharmacological blockers of Cdk have non-specific effects [34], we examined whether blockade of

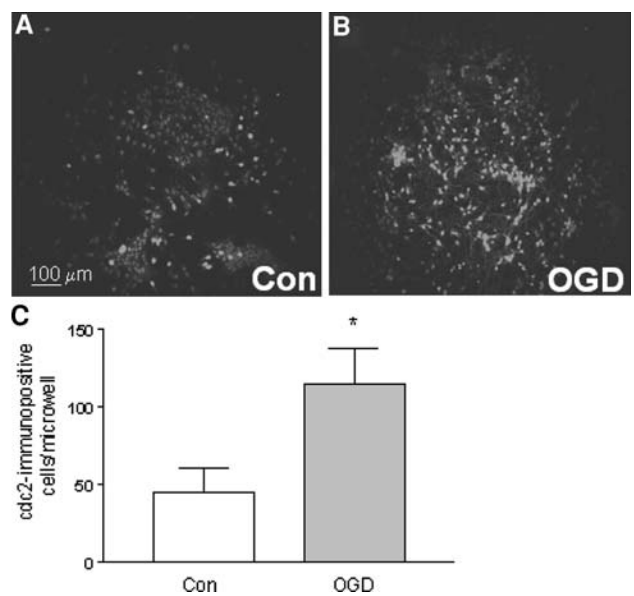

Fig. 1 Increased cdc2-immunoreactivity was observed in septal cultures subjected to 5 h OGD. (**a**) Sample photograph of cdc2-immunoreactive neurons in a microwell from a 72-well terasaki culture dish (Control). (**b**) cdc2-immunoreactive neurons at 24 h after the end of OGD. (**c**) Plots of cdc2-immunoreactive cells in each microwell in a 72-well culture dish. Hoechst staining was used to determine the total number of cells in each well and each microwell contained ~1,000 cells. Data, presented as mean ± SEM, are collected from 2 separate culture dishes from different experiments and seedings with 36 microwells in each condition

retinoblastoma protein would protect neuronal process disintegration after OGD by overexpressing a non-phosphorylatable form of Rb. At 24 h after the end of OGD, there was little difference in survival between cells overexpressing Rb-wild type (Rb-wt) or Rb mutant (RbΔK11) (68.1 ± 5% and 70.4 ± 4.1%, respectively; Figs. 2 and 3) when compared to GFP-only controls (54.3 ± 4.4%). The morphological criterion for neuronal survival was compared with a histological criterion, exclusion of propidium iodide (PI, 2 μg/ml), a marker for dying cells [32]. Surviving cells with intact processes did not stain for PI. There was minimal damage in non-stressed controls with 82% of Rb-wt and 89% of RbΔK11 showing intact processes. At 48 h, when compared to the survival of GFP only cells (22.8 ± 3.8%), cells expressing RbΔK11 showed more robust protection (50.4 ± 4.5%; $P < 0.05$ vs GFP) than Rb-wt (27.2 ± 4.7%). Cell survival in the Rb-wt expressing non-stressed neurons was 79% whereas in RbΔK11 it was 81%. This result is consistent with other published articles where pharmacological inhibitors of cdk4/6 are neuroprotective in ischemia [3, 8]. The transient protective effect of overexpressing Rb-wt observed at 24 h may have been due to competition for the kinases that phosphorylate Rb. Such competition might slow the rate at which Rb molecules achieve sufficient phosphorylation to block their inhibition of cell cycle entry.

Overexpression of Cdk5-Wild Type but not Cdk5-Dominant Negative Prevents Disintegration of Neuronal Processes Following OGD

While Cdk5 is not directly involved in cell cycle control functions, one of the substrates of p25/Cdk5 is Rb [35, 16] which is a key regulator of cell cycle progression. To evaluate the role of Cdk5 following oxygen–glucose deprivation (OGD), cultures were exposed to OGD for 5 h. At 24 h after the end of OGD, 49.2 ± 3.2% of cells expressing only GFP survived the stress whereas 84 ± 4.31% of neurons expressing Cdk5-wild type (Cdk5-wt) survived the stress ($P < 0.05$ vs GFP; Fig. 4). 61.1 ± 4.4% of cells expressing Cdk5T33 (Cdk5-dominant negative) survived OGD. Non-stressed controls had a survival rate of 86% (Cdk5-wt) and 82% (Cdk5-dn). As described in the earlier experiment, surviving cells with intact processes did not stain for PI. At 48 h after OGD, the majority of GFP-only neurons had lost their processes with shrunken cell bodies (26.82 ± 3.8 %).

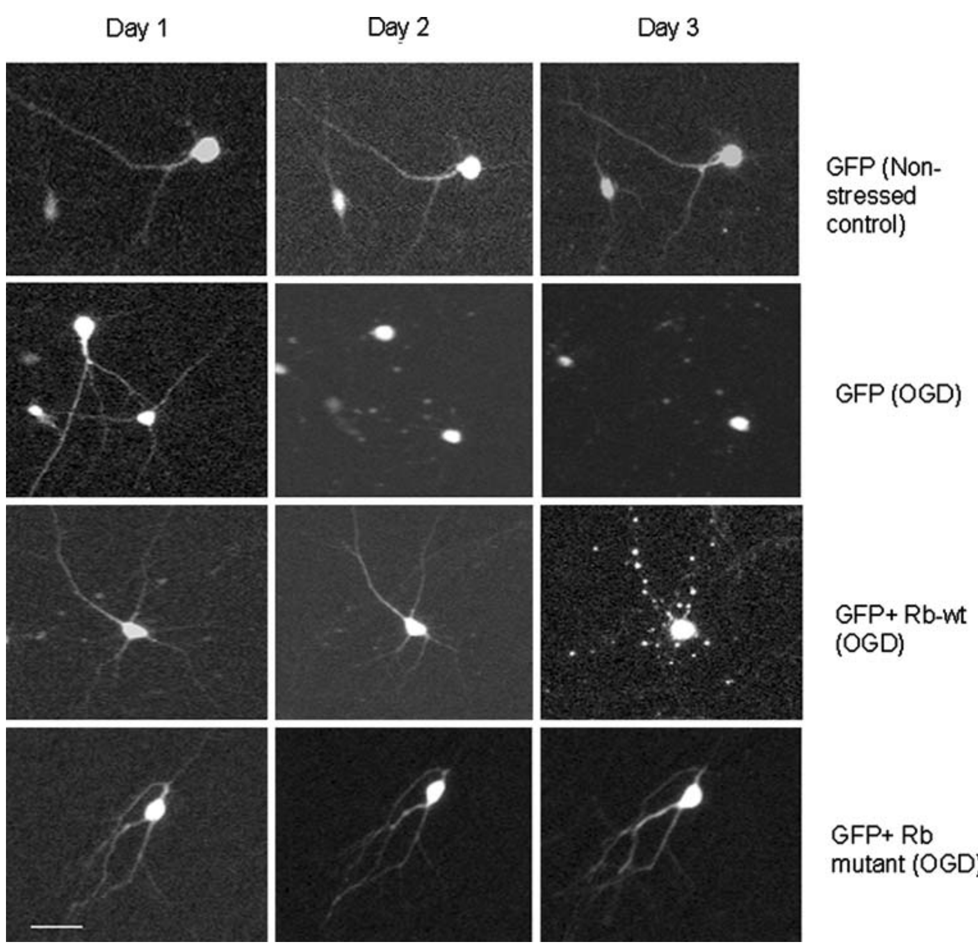

Fig. 2 Overexpression of Rb-mutant (RbΔK11) but not Rb-wild type (Rb-wt) prevented loss of neurites following OGD. Panels show sequential fluorescence micrographs of representative septal neurons transfected with GFP alone (upper 2 panels) or with GFP+Rb wild type (Row 3), or GFP+Rb mutant (last row). Images were collected before the stress in control medium on day 1 followed by 5 h OGD (see OGD protocol). Same neurons were re-imaged 24 h after the stress (day 2), then returned to normal conditioned medium and imaged again 48 h after OGD (day 3). Non-stressed controls received media change but were maintained in normal medium throughout. Results from this experiment are plotted. Scale bar = 50 μm

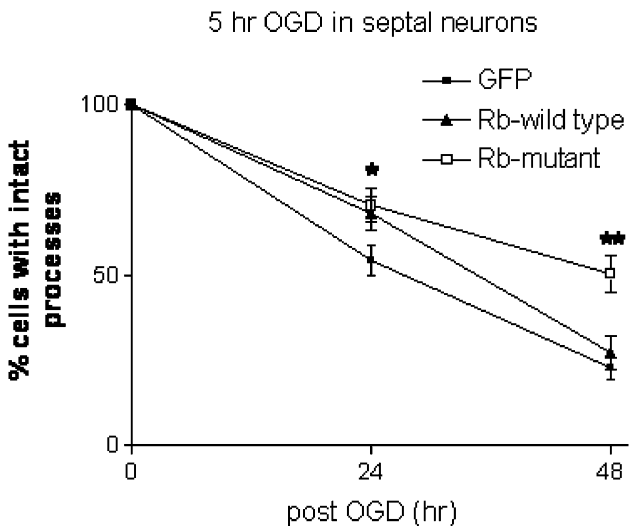

Fig. 3 Overexpression of Rb mutant but not Rb-wt decreased loss of neurites following 5 h OGD in septal neurons. Images of transfected neurons were collected immediately before OGD and the total number of intact neurons was set as 100%. Same neurons were imaged 24 and 48 h after the end of OGD. Neurons counted as surviving (mean ± SEM) remained intact morphologically and did not stain for propidium iodide (**P* < 0.05 vs GFP only at 24 h; ***P* < 0.05 vs GFP only and Rb-wt at 48 h) and is plotted as percent. Each group included between 250 and 340 transfected neurons from 2 separate platings using 72-microwell culture dishes. Non-stressed controls were washed but maintained in normal medium throughout (data not shown)

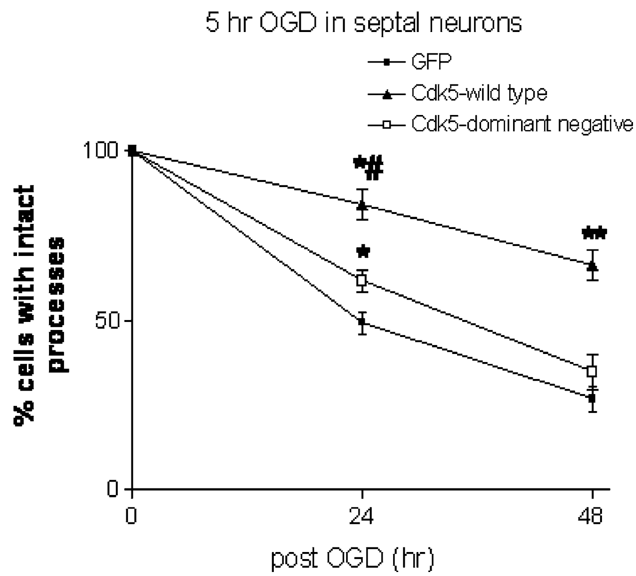

Fig. 4 Overexpression of Cdk5-wild type (Cdk5-wt) but not Cdk5-dominant negative (Cdk5T33) decreased loss of neurites following 5 h OGD in septal neurons. As in Fig. 2, images of transfected neurons were collected immediately before OGD and the same neurons were imaged 24 and 48 h after the end of stress. Surviving neurons (mean ± SEM) remained intact morphologically (**P* < 0.05 vs GFP only, #*P* < 0.05 vs Cdk5-dominant negative, both at 24 h; ** vs GFP only and Cdk5-dominant negative at 48 h) and is plotted as percent. Each group included between 130 and 165 transfected neurons from 2 separate platings. Non-stressed controls were washed but maintained in normal medium throughout (data not shown)

Interestingly, 66 ± 3.2% of Cdk5-wt neurons survived ($P < 0.05$ vs GFP) but not Cdk5T33 (34.70 ± 5.34%). There was 6% cell death in non-stressed Cdk5-wt and 8% in Cdk5-dn when compared to 24 h.

Discussion

Our results indicate that there is activation of the cell cycle machinery in septal neuronal cultures after OGD as evidenced by an increase in cdc2-immunoreactive neurons. While this finding is consistent with other studies which report increases in other cell cycle related proteins in neurons after ischemic injury, there are differences in the literature regarding the expression of such proteins. Katchanov et al. [4] report an increase in cyclin D and cdk2 protein levels in primary cortical neurons after OGD. Several studies have also reported an increase in cyclin D protein levels in neurons in vivo following middle cerebral artery occlusion [2, 3, 4, 36]. Jin et al. [6] report an increase in cyclin H, but no increase in cyclin D or cdc2 levels using a 4-vessel occlusion model of ischemia in rats. Small et al. [37] found a decrease in cyclin D in a rat model of global ischemia. It is unclear whether the differences are due to the ischemic model used or the severity of stress.

The protective effects found when overexpressing non-phosphorylatable Rb are consistent with the hypothesis that phosphorylation of Rb triggers events that lead to neuronal damage and process disintegration during oxygen–glucose deprivation stress. Since phosphorylation of Rb is a critical early event in cell cycle activation, this finding fits with the hypothesis that activation of cell cycle related molecules is involved in neuronal death during ischemia-like stress. Overexpression of the non-phosphorylatable Rb would be expected to reduce cell cycle activation, and so its protective effect is consistent with studies where blockade of cell cycle related kinases was neuroprotective in ischemia-like models [3, 4, 8]. Loss of p16ink4a and p27kip1, endogenous Cdk inhibitors occur in striatal neurons after ischemia in mice [4], increased phosphorylation of Rb [38], and increased expression of cyclin D1 [39, 2] after ischemia in rats are all consistent with the hypothesis that activation of a Cdk-Rb pathway occurs after ischemia. Moreover the cell cycle initiating transcription factor, E2F1, is upregulated following stroke [3] and E2F1-deficient mice are resistant to ischemia [40] consistent with a role of at least partial cell cycle activation in neuronal damage from ischemia. Also, olomoucine, a blocker of Cdks 1, 2 and 4, protects primary cortical neuronal cultures from OGD-induced cell death [4].

Cdk5's major functional roles are thought to involve the cytoskeleton (see [41] for review), but it does phosphorylate Rb in a neuronal cell line [16], and damaging effects of Cdk5 activated by p25 have been implicated in ischemia [42, 43]. Thus one might predict that Cdk5-dependent Rb phosphorylation would enhance cell cycle activation and so increase neuronal damage following cellular stress. But the role of Cdk5 in neuronal damage is complex, and may depend on whether Cdk5's kinase activity is activated by p35 (protective) or p25 (toxic), and/or whether Cdk5 remains within the cytoplasm (protective) or enters the nucleus (toxic, [44, 45]). We found robust protection of neurites by overexpression of Cdk5-wt and little effect of expressing a kinase-inactive Cdk5. The protection afforded by Cdk5 overexpression might be due to phosphorylation of p35 by Cdk5. This would target p35 to the proteosome and reduce the formation of p25 via cleavage of p35 by calpains [46, 47, 48]. Alternatively, the protective effects of Cdk5-wt overexpression may be mediated by mechanisms independent of Rb and the cell cycle. Pharmacological inhibitors of Cdk5 abolish a protective effect of aspirin reported in rat spinal cord cultures after hypoxia/reoxygenation [49]. Cdk5 could also protect by phosphorylating and thereby negatively regulating other kinases such as Mek1 [50] and c-jun N-terminal kinase 3 (JNK3) [51]. Alternatively, Cdk5 may promote post-stress neuronal survival by increasing Akt activity through the neuregulin/PI3-K signaling pathway [52]. Inhibition of Cdk5 reduces endocytosis of NR2B [53], an NMDA receptor subunit that contributes to excitotoxic cell death following ischemic injury, so it is conceivable that overexpression of Cdk5 may protect by reducing surface expression of NMDA receptors.

Our results indicate that both Cdk5 and cell cycle activation are likely to have important (but opposite) effects on septal neuronal survival following ischemia-like stress. Unfortunately there are no currently available pharmacological inhibitors that robustly inhibit the cell cycle kinases without at least partially inhibiting Cdk5. This and other studies show that activation of the cell cycle and its associated kinases is damaging during ischemia, but this study indicates that Cdk5 activity can be protective under some ischemia-like stress conditions. New agents that selectively inhibit cell cycle activation while not inhibiting (or instead activating) Cdk5 might be beneficial in reducing neuronal damage from ischemia.

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